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Glycosylation of Antibody Molecules: Structural and Functional Significance

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The IgG antibody molecule is a structural paradigm for members of the immunoglobulin superfamily. A majority of these molecules are glycoproteins and collectively they account for ~70% of molecules currently undergoing development for possible in vivo therapeutic application. Whilst the oligosaccharide moiety of the IgG molecule accounts for only 2–3% of its mass, it has been shown to be essential for optimal activation of effector mechanisms leading to the clearance and destruction of pathogens. This suggests that glycosylation fidelity is an essential requirement of the IgG molecule and that it may be so for other recombinant glycoproteins produced by in vivo or in vitro techniques. Numerous studies have shown that whilst the defining biological activity of a glycoprotein molecule may not be dependent on its glycosylation, other essential characteristics are altered in aglycosylated forms, e.g. stability, pharmokinetics, antigenicity [1–3].

The IgG molecule is composed of three globular protein moieties, two Fabs and an Fc, that are linked through a flexible 'hinge' region that allows freedom for multiple spatial orientations of the globular moieties with respect to each other. A flexible upper hinge region provides mobility for the Fab regions and allows the paratope of each to bind its complementary epitope. A flexible lower hinge region similarly allows Fc mobility and accessibility within antigen/antibody complexes to engage one of a variety of effector activating ligands, e.g. Fcγ receptors, the C1 component of complement. A core hinge section is rich in proline and cystine residues, that form inter-heavy chain disulphide bridges, and has a rigid secondary structure.

Studies attempting to correlate physicochemical parameters with function were interpreted to suggest that the segmental flexibility of the hinge region

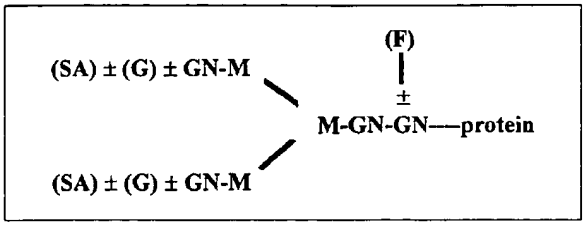

Fig. 1. The core carbohydrate moiety of the complex form of oligosaccharides is represented by the sugar residues in open type. The possible outer arm residues are bracketed. All possible combinations are observed. SA = Sialic acid; G = galactose; GN = N-acetylglucosamine; M = mannose; F = fucose. N-linked attachment of oligosaccharide occurs on the amide side chain of the Asn-x-Ser/Thr sequon (x ≠ Pro); the Ser/Thr residue forms hydrogen bond(s) with the amide group in order to activate it for attachment to the primary N-acetylglucosamine residue of the dolichol intermediate, by oligosaccharyltransferase.

was directly related to the ability of an IgG molecule to activate complement; rather than indirectly by allowing access to interaction sites in the C_H2 and/or C_H3 domains for effector ligands [4]. The validity of these conclusions has recently been re-evaluated by the application of protein engineering techniques in an attempt to introduce rational structural changes predicted to affect biological activity. The results of these studies demonstrate the necessity for Fc glycosylation and that protein/oligosaccharide interactions determine the generation of a structure that is permissive of Fc-ligand recognition and activation, while failing to confirm a primary role for the hinge region. Our studies suggest that whilst the oligosaccharide moiety may not contribute directly to ligand binding, except for mannan-binding protein, it does exert a subtle influence on protein tertiary and quaternary structure that is essential to 'wild type' activity. Consequently, Fc-ligand recognition, and hence biological activity, may be modulated by judicious replacement of amino acid residues that contribute to non-covalent protein/oligosaccharide interactions.

Antibody Glycosylation

Human antibody molecules of the IgG class have N-linked oligosaccharide attached at the amide side chain of Asn297 on the β-4 bend of the inner (Fx) face of the C_H2 domain of the Fc region [5]. The oligosaccharide moiety is of the complex biantennary type having a hexasaccharide 'core' structure (GlcNAc2Man3GlcNAc) and variable outer arm 'non-core' sugar residues, such as fucose, bisecting N-acetylglucosamine, galactose and sialic acid (fig. 1).

Thus, a total of 36 structurally unique oligosaccharide chains may be attached at each Asn297 residue. It is anticipated that glycosylation can be asymmetric so that an individual IgG molecule may have different oligosaccharide chains attached at each of the Asn297 residues within the Fc region such that whilst the heavy chain synthesised within a single antibody-secreting cell may be homogeneous in its amino acid sequence glycosylation can result in the production of $(36 \times 36)/2 = 648$ structurally unique IgG molecules or glycoforms; NB: the total number of combinations is divided by two because of the two-fold symmetry of the molecule. Analysis of monoclonal and polyclonal IgG demonstrates the presence of all the predicted oligosaccharide species, however, disialylated oligosaccharides may be absent or present at a very low level [6]. With the additional possibility of the presence of complex N-linked oligosaccharide in the Fab region, it is apparent that glycosylation is a post-translational modification that can introduce a very significant structural and, possibly, functional heterogeneity into the IgG molecule. The presence of additional glycosylation sites within the heavy chains of the other Ig isotypes means that the possible number of glycoforms may be orders of magnitude higher [7, 8].

The Fc glycosylation site is a conserved feature for all mammalian IgGs investigated and glycosylation occurs at a homologous position in human IgM, IgD and IgE, molecules, but not in IgA. Human IgM, IgA, IgE and IgD molecules bear additional N-linked oligosaccharide moieties attached to the constant domains of the heavy chains and IgA subclass 1 (IgA1) and IgD proteins also bear multiple O-linked sugars in their extended hinge regions, attached to hydroxyl groups of serine and threonine residues. It has been estimated that ~30% of polyclonal IgG molecules also bear an oligosaccharide moiety within the Fab region. Since the sequences of the constant region of κ and λ light chains and the C_H1 domain of the heavy chain do not include a glycosylation sequon, the oligosaccharide of glycosylated Fab regions is due to attachment within either the V_L or V_H sequences. Analysis of the DNA sequences of 83 human germline V_H gene segments revealed five that encoded potential glycosylation sites, however, none of these sequons were observed in 37 V_H protein sequences – detailed analysis to determine whether the germline gene from which these proteins were derived did encode a glycosylation sequon was not attempted. Fifteen of the 37 protein sequences did have potential glycosylation sequons which, it would appear, have resulted from somatic mutation and antigen selection [7, 8]. It has been demonstrated that the structure and function of Fab oligosaccharide can depend on the site of attachment. Thus, monoclonal murine anti-dextran antibodies with a single oligosaccharide attachment site at residues 54, 58 or 60 in complementarity-determining region 2 (CDR2) were shown to have differing antigen-binding activities [9]. A monoclonal human polyreactive autoantibody, secreted by a heterohybridoma cell

line was shown to be glycosylated on both the V_L and V_H regions; the V_H glycosylation site was at residue 75 in framework 3, adjacent to CDR3 [10].

Structural Consequences of IgG Glycosylation

Whilst glycosylation of the IgG/Fc is essential for optimal expression of effector activities mediated through FcγR and the C1 component of complement direct interaction of the oligosaccharide moiety with these effector ligands has not been demonstrated. Recently, it was reported, however, that in agalactosylated IgG the oligosaccharide moiety 'flips' out of the inter-C_H2 space and the terminal N-acetylglucosamine residues become available to bind and activate mannan-binding protein [11], and consequently the classical complement cascade. By contrast, these residues are not available to the lectin *Bandeireae simplicifolia* II in the native form of agalactosylated IgG but become so on denaturation. Resolution of structure for the oligosaccharide chains in x-ray crystallography demonstrates that it is not freely mobile and has definite conformation. From its attachment point at Asn297, it 'runs forward' towards the C_H2/C_H3 interface region and it is estimated that 82 non-covalent interactions between core sugar residues and the outer arm residues of the $\alpha[1\rightarrow6]$ arm may be possible [12]. Together with the sugars of the $\alpha[1\rightarrow3]$ arm, the oligosaccharide fills the available volume between the C_H2 domains. It may be anticipated, therefore, that whilst interactions with the Fx face of the protein impose structure on the oligosaccharide chain there is a reciprocal influence of the oligosaccharide on the protein structure.

The structural and functional consequences of Fc glycosylation can be assessed by comparison of glycosylated and aglycosylated forms of IgG. The latter can be generated by production in *Escherichia coli*, growing IgG producing cells in the presence of the glycosylation inhibitor tunicamycin or by protein engineering of the glycosylation sequon. It should be appreciated that IgG produced in *E. coli* or in the presence of tunicamycin will have an asparagine residue at 297 whilst site-directed mutagenesis can introduce any chosen amino acid residue; in the present case, alanine. A more subtle approach is to isolate homogeneous glycoforms for structural and functional studies or to replace individual or combinations of amino acid residues that make contacts with sugar residues. The latter approach may allow a detailed understanding of the oligosaccharide/protein interactions in this molecule, the 'rules' of template direction and its effect on the type of oligosaccharide attached and the generation of mutant molecules with new profiles of biologic function.

A small, localised protein structural change has been detected for aglycosylated human chimeric IgG3 and its Fc fragment by ^{1}H-NMR. Previous studies

had allowed assignments for each of the five histidine residues, and their distribution through the Fc makes them suitable probes for detection of localised structural change. Such a change was reported for His268 which is in the vicinity of both the carbohydrate attachment site and the lower hinge binding site on IgG for Fc receptors [13]. A similar spectral difference was observed between a glycosylated IgG1 Fc fragment and the aglycosylated form produced as a recombinant protein in *E. coli* [14]. A structural difference between the lower hinge regions (residues 234–237) of glycosylated and aglycosylated IgG was inferred from the different papain cleavage profiles obtained for glycosylated and aglycosylated mouse IgG2b. Whilst a single cleavage point at residue 229 was observed for the glycosylated protein, the aglycosylated mutant was cleaved heterogeneously at residues 228, 234, and 235 [15].

Recent ^{13}C-NMR studies have provided direct evidence of differing structural dynamics for the lower hinge residues of glycosylated and aglycosylated mouse IgG2b [Kato, Lund and Jefferis, unpubl.]. Significant differences are revealed when thermodynamic parameters are determined from data obtained from differential scanning microcalorimetry of glycosylated and aglycosylated mouse IgG2b-Fc [Tischenko, Lund and Jefferis, unpubl.]. Differences are observed in both the C_H2 and C_H3 domains and the free energy of stabilisation of the C_H2 domain is decreased. An attempt to monitor structural differences between glycosylated and aglycosylated human IgG3 through altered epitope expression, employing a panel of > 30 mouse monoclonal anti-human Fcγ antibodies, did not detect any loss of expression or obvious reduction in affinity.

Functional Consequences of Asn297 Glycosylation

Since it has been consistently demonstrated that glycosylation is essential for optimal expression of FcγR- and C1-mediated effector functions, it may be anticipated that biological activity may vary between differing glycoforms. Most studies have compared differences between natural forms of IgG and their aglycosylated or agalactosylated counterparts; however, we have added the approach of generating mutant proteins in which residues reasoned to participate in oligosaccharide-protein interactions have been replaced.

A wide range of effector cells are activated by IgG/antigen immune complexes through interactions with cellular receptors for the Fc region of the gamma chain, FcγR. Three types of human Fc receptors (FcγRI, FcγRII, and FcγRIII) have been defined, by gene cloning and sequencing, that are differentially expressed on a variety of cell types; additionally FcγR may be induced or their expression up-regulated following cellular activation. The IgG isotype specificity of the FcγR suggests that recognition is correlated with

primary amino acid sequence. An earlier prediction that the lower hinge residues 234–237 (-Leu-Leu-Gly-Gly-) in particular might correlate with FcγRI recognition appeared to be confirmed by protein engineering studies with the demonstration that replacement of any one of these residues in mouse/human chimeric IgG3 affected recognition by all three human FcγR [16, 17]. It was proposed, therefore, that the three Fc receptors are recognised by overlapping, non-identical ligand-binding sites. This appears rational for a family of receptors that are evolutionarily related and exhibit a high degree of sequence homology.

Other structural features are also determinants for recognition since replacement of Pro331 by serine, the amino acid residue present at this position in IgG4, reduces the binding affinity for IgG1 and IgG3 by an order of magnitude [18]; these residues are within 11 Å of the lower hinge. The FcγRII receptor is polymorphic and the allelic forms are designated as FcγRIIa-H131 and FcγRIIa-R131 to indicate that a histidine/arginine interchange at residue 131 is critical to recognition of human IgG2 molecules [19]. Thus, monocytes of homozygous H/H131 individuals were found to internalise IgG2-opsonized erythrocytes more efficiently than cells from R/R131 individuals. Since the lower hinge region of IgG2 molecules is radically different from that of IgG1 and IgG3, it is apparent that the recognition site for FcγRII depends on structure outside this region.

The amino acid replacement studies suggest that FcγR recognition is dependent on a precise molecular architecture and that subtle structural changes have a dramatic effect on biological function. This conclusion is further supported by the demonstration that aglycosylated human chimeric IgG3 has a reduced interaction with all three Fc receptors [16, 17, 20]. Whilst hapten-derivatised red blood cells could still be sensitised with this antibody to trigger superoxide production by U937 cells, stimulated with γ-interferon, higher levels of sensitisation were required compared to glycosylated IgG3 [20]. The aglycosylated IgG3 was not recognised by human FcγRII expressed on K562 and Daudi cells [21] and rosette formation mediated through FcγRIII, expressed on human NK (natural killer) cells, was reduced to 40% of that obtained for glycosylated IgG3, whereas antibody-dependent cellular cytotoxicity (ADCC) was essentially abolished [17]. Comparative studies of a glycosylated and an aglycosylated humanised anti-CD3 antibody suggest that the altered biological activities of aglycosylated IgG may be exploited for some in vivo applications. In the model investigated the glycosylated IgG was able to effect immune modulation and was immunogenic; probably due to its ability to activate T cells following interactions with appropriate FcγR-expressing cells. By contrast, the aglycosylated antibody was not immunomodulatory, was less immunogenic and had a longer half-life [22].

Attempts to evaluate the contribution of outer arm sugars to biologic function have concentrated mostly on glycoforms differing in galactose content. In a sustained investigation of the EBV-transformed lymphocytes secreting anti-D antibody, it has been shown that antibody with a high galactose content (>70% digalactosyl IgG) was more effective than antibody with a low galactose content (10% agalactosyl and 50% monogalactosyl IgG) in FcγRI- and FcγRIII-mediated cellular lysis (ADCC) [23]. An evaluation of the contribution of galactosylation to FcγRI recognition was made by comparison of the ability of a low galactose (<20% galactosylated) and a fully galactosylated form of an IgG4 Fc to inhibit superoxide generation through mouse/human chimeric IgG3, no difference was detectable in this system [24]. A minimal reduction in FcγR and a 2-fold reduction in C1q binding for agalactosyl IgG relative to the galactosylated form has been reported [25].

Considerable clinical experience has been gained with the humanised monoclonal antibody Campath-1H and its promise requires optimisation of control and efficiency of production. The product of rat YO, chinese hamster ovary (CHO) and mouse NSO cells has been evaluated for glycosylation and ADCC activity [26]. Interestingly, the rat cells were demonstrated to secrete IgG with relatively high levels of bisecting GlcNAc and to be the most active of the three products in ADCC, leading to the conclusion that this glycoform may have significant biologic advantage. The product of the NSO cells was reported to be underglycosylated. The final conclusion was that the cell type was a more important parameter than the culture conditions, at least for medium with and without added serum. It should be noted, however, that the method of culture used for each cell type was significantly different; the YO cells were grown in roller bottles, the NSO cells in shaking flasks and the CHO cells in hollow fibre bioreactors. In our experience [27], these differences in the method of culture could account, in large part, for the differences in glycoform profiles observed. The influence of outer arm sugars was evaluated for Campath-1H antibody following exposure to neuraminidase and β-galactosidase; removal of low levels of sialic acid had no effect on ADCC or complement-mediated lysis (CML), however, whilst removal of galactose was without effect on ADCC, it resulted in ~50% reduction in CML activity [28].

The essential requirement for protein/core-oligosaccharide interactions with a biantennary-type oligosaccharide is suggested from studies of a chimeric mouse-human IgG1 antibody produced in Lec-1 cells which are incapable of processing high mannose forms of oligosaccharide [29]. The antibody product having a high mannose oligosaccharide attached at Asn297 was incapable of complement-mediated hemolysis and deficient in C1q and FcγR1 binding. In contrast a chimeric mouse-human IgG1 antibody produced in yeast cells, with presumed incorporation of high mannose forms of oligosaccharides at Asn297,

maintained the ability to trigger ADCC through human FcγRIII [30]. The importance of C_H2 domain protein/core-oligosaccharide interactions in IgG is emphasised by the demonstration that recognition by Fcγ receptors can be modulated in mutant proteins in which core oligosaccharide contact residues have been replaced. Thus, replacement of Asp265, a contact residue for the primary GlcNAc residue of the core oligosaccharide, resulted in reduced recognition by human FcγRI and human FcγRII. By contrast, replacement of non-core contact residues Lys246, Asp249 by Ala and Glu258 by Asn was without effect on recognition for these receptors, a finding consistent with the view that the interactions with GlcNAc and Gal residues of the Manα(1→6) arm are not essential for maintenance of recognition by human FcγRI and FcγRII [24].

The biological half-life of a recombinant glycoprotein is a vital property determining in vivo efficacy and the economics of treatment. Studies of blood clearance of glycosylated and aglycosylated mouse/human chimeric IgG1 in mice demonstrated accelerated clearance for the aglycosylated form but with similar half-lives. Since the half-life of IgG1 in humans is ~23 days but measured as 5 days in this model it is difficult to draw a definitive conclusion. Catabolism of aglycosylated mouse IgG2b was evaluated in a rat model and shown to be cleared more rapidly than the glycosylated form and it was concluded that the increased catabolism occurred in the extravascular space [31]. The plasma half-lives and bioavailability of human anti-D antibodies secreted by Epstein-Barr virus (EBV)-transformed human B cells, cultured in hollow-fibre bio-reactors, have been evaluated in vivo in comparison with polyclonal anti-D isolated from immunised volunteers [32]. The half-lives of an IgG1 and an IgG3 monoclonal anti-D antibody were 22.2 and 10.2 days, respectively, compared to 15.6 days for polyclonal anti-D IgG. The half-life of polyclonal anti-D IgG was dependent on the proportions of IgG1 and IgG3 present in the preparation. Studies of mutant mouse IgG1 proteins have been interpreted to localise the site controlling catabolism to the inter C_H2/C_H3 region and to demonstrate modulation of the half-life [33].

Evaluation of a panel of 28 mutant mouse IgG2b proteins, each with a surface accessible amino acid replacement, for C1q binding and C1 activation correlated recognition with the presence of the wild-type residues lysine, glutamic acid and glutamic acid at 318, 320 and 322 [34]. A contrary result has been reported for mouse/human chimeric IgG1 antibody with the demonstration that replacement of glutamic acid 320 was without effect on CML, however, C1 activation was abrogated following amino acid replacements in the lower hinge region [35]. This is consistent with the observation that a Pro→Ser replacement at residue 331 in IgG1 and IgG3 results in a reduced capacity to trigger complement lysis [36]. One of the mutant proteins produced by Duncan

and Winter [34] was Asn → Ala, 297 which results in the production of aglycosylated mouse IgG2b. This protein had a 3-fold reduced capacity to bind human C1q and a much reduced ability to trigger lysis of target cells with guinea pig complement through the classical complement cascade. Similarly, an aglycosylated mouse/human chimeric IgG1 was shown to retain some ability to trigger lysis of target cells by human complement but with a 7- to 8-fold higher antibody concentration requirement than for the glycosylated wild type IgG1 [37]. These data suggest a similarity in the molecular requirements for FcγR and C1 recognition and that glycosylation is essential for generation of a quaternary structure expressing these ligand binding sites.

The role of outer arm sugars in C1 mediated lysis has been investigated for galactosylated and agalactosylated IgG, produced following exposure to β-galactosidase, with an observed 2-fold higher activity for the galactosylated form [25]. Confirmation of the importance of correct glycosylation is provided by study of a human-mouse chimeric IgG1 molecule produced in yeast cells and anticipated to have high mannose type oligosaccharide attached at Asn297 [29]. The IgG1 product was unable to activate C1 to trigger human complement mediated lysis of targets whilst the same chimeric IgG1 construct expressed in rodent cells (Sp2/0) was effective. A direct role for the oligosaccharide moiety in activating the complement cascade is apparent for the lectin mannan-binding protein which can function as a surrogate C1 component. The specificity of mannan-binding protein is for mannose and N-acetylglucosamine residues, and it has been shown that it can access and bind to terminal N-acetylglucosamine residues exposed on agalactosyl IgG [11].

Much interest has been generated by the observation of a deficit in IgG galactosylation in patients with rheumatoid arthritis (RA) and some other inflammatory diseases, including tuberculosis and Crohn's disease [38]. Another feature of RA is the presence in the blood of rheumatoid factor (RF) autoantibodies having specificity for epitopes in the Fc region of IgG. Since RFs are, typically, of IgM or IgG isotype, the immune complexes formed have the potential to trigger effector functions through IgG-mediated pathways or a combination of IgG and IgM pathways. The chronic inflammatory reactions resulting are thought to contribute erosive damage in this disease. A dominant specificity of RFs is for an epitope localised to the area of contact and interaction between the C_H2 and C_H3 domains. This specificity overlaps with that of Staphylococcal protein A and the binding of a majority of RFs to IgG can be inhibited by Staphylococcal protein A [8, 39]. It has been speculated that terminal galactose or sialic acid residues on the α[1 → 6] arm of the oligosaccharide may be accessible to RFs and influence recognition and binding affinity, with a consequent effect on the nature and size of immune complex formed. A galactose residue on the α[1 → 6] arm is resolved on x-ray crystallography

and possible non-covalent contacts identified. It has been argued, therefore, that this galactose residue occupies a lectin-like pocket that will be exposed in agalactosylated IgG and may contribute to altered IgG antigenicity, e.g. reactivity with RFs [38]. Alternatively, the mannose-binding protein provides a route by which agalactosylated IgG could trigger the inflammatory reactions seen in RA independently of RFs [11].

A study of the reactivity of 16 monoclonal RFs generated from synovial tissue lymphocytes with IgG of differing galactose content (18–86%) yielded ambiguous results. Five RFs reacted more avidly to IgG of low galactose content, 6 were not influenced by galactose content and one bound more avidly to IgG of high galactose content [40]. A comparison of the binding of polyclonal and monoclonal RFs to glycosylated and aglycosylated chimeric mouse/human IgG proteins of each of the subclasses detected no differences for IgG1, IgG2 and IgG4 proteins, however, RFs reactive with IgG3 proteins reacted more avidly with aglycosylated IgG3 [41]. In a companion study, some monoclonal RFs were found to bind aglycosylated IgG4 less well than glycosylated IgG4 (2- to 5-fold), suggesting that the carbohydrate moiety is important in establishing their binding epitope in the C_H2 domain [42]. An interesting difference between the latter two studies was that for one the source of monoclonal RF was serum of patients with Waldenströms macroglobulinemia [41] and for the other EBV-transformed synovial tissue lymphocytes of RA patients. Given the parallelism between RF and Staphylococcal protein A binding to IgG, it is pertinent to note that there is only one report of a minimal effect of glycosylation on the binding of Staphylococcal A to IgG. An interesting recent study demonstrated isotype regulation mediated through the generation of auto-anti-isotype antibodies (RFs) during the course of an immune response to influenza virus. A series of RFs were established as monoclonal antibodies and demonstrated to effect immune deviation in vivo. One of these RFs, a monoclonal IgA RF, specific for mouse IgG2b bound the aglycosylated protein poorly [43].

On complexing with polyvalent antigen, IgM is able to initiate the classical complement cascade, following binding of C1q molecule to the C_H3 domain; the equivalent of the C_H2 domain of IgG. Amino acid replacements within glycosylation sequon 402–404 [44] of the C_H3 domain of mouse IgM results in a 3- to 25-fold decrease in the capacity to effect CML of target cells by guinea pig complement. This lowered activity could be due, at least in part, to an observed 4- to 8-fold reduction in assembly of the monomeric subunits into pentameric and hexameric IgM molecules. Replacement of residue 406 (Ser→Asn), analogous to core contact residue 301 in IgG, resulted in a 50-fold decrease in the capacity of mouse IgM to trigger lysis through guinea pig complement [45]. These data suggest that interactions between amino acid

residues and core sugar residues of the oligosaccharide attached at Asn-402 of the IgM molecule may be important for the formation of the C1-binding and activation site.

Factors Influencing Glycosylation Hybridoma, and Recombinant Immunoglobulin Molecules

Regulatory authorities demand exhaustive testing of monoclonal antibodies that might be applied for in vivo diagnostic or therapeutic purposes. If approved, a similar demand for the demonstration of product consistency is made. The parameters that should be analysed, in vitro, include isotype, subclass, affinity, microheterogeneity, molecular weight, primary and secondary structure, structural integrity, specificity, glycosylation profile, biological potency. Subsequently, the product would be evaluated for pharmacological, toxicological, biodistribution and half-life in vivo [3]. Functional studies of recombinant human proteins have established that the form of the oligosaccharide moiety attached at a specific glycosylation site should be the same as that attached to the natural molecule. Regulatory authorities require authentic and consistent glycosylation of molecules that may be applied in vivo, therefore, animal cells are preferred to other systems for their production. The biotechnology industry has concentrated on development of production protocols employing CHO cells for all recombinant human glycoproteins demonstrating that its glycosylation machinery is catholic and that the polypeptide chain has a major influence on the type of oligosaccharide attached. However, CHO cells do not satisfy industrial economic requirements for the production of antibodies and so there has been a resurgence of interest in NSO cells that are derived from an antibody secreting plasmacytoma. A recent study also employed the rat plasmacytoma line YO and demonstrated its product to have a natural glycosylation profile that included the presence of bisecting GlcNAc residues [26]. For any given cell type, glycosylation of antibody products remains a variable dependent on numerous parameters that include, the method of cell culture, the supply of nutrients, removal of metabolic products, when the protein is harvested and a subtle influence of the polypeptide chain on outer arm sugar heterogeneity. A further concern is the possibility that mutant clones may arise during extensive and continuous culture with the emergence and overgrowth of a sub-clone secreting structurally and functionally aberrant molecules. The reality of this concern is demonstrated by the isolation of multiple sub-clones of CHO cells each of which expresses an altered profile of glycosyltransferases and consequently secretes glycoproteins with unique glycoform profiles [46].

Experience in the production of mouse/human chimeric antibodies in J558L cells demonstrated significant differences in galactosylation depending on whether it was produced in shallow culture, hollow fibre bioreactor or in vivo, as ascitic fluid. Of particular concern is the production of variable proportions of molecules bearing additional galactose residues in $\alpha[1\rightarrow3]$ linkage to normal galactose sugars. This results from the activity of an endogenous $\alpha[1\rightarrow3]$-galactosyltransferase. Gene expression for this enzyme is depressed in humans and higher primates with the result that it constitutes an immunogenic structure and it has been estimated that 1% of circulating human IgG is 'anti-Gal' antibody [47] and its presence can be readily demonstrated in an ELISA [48]. Although the CHO cell line expresses an $\alpha[1\rightarrow3]$ galactosyltransferase, there appears to be only one documented instance in which it has been demonstrated to be active in the addition of Gal $\alpha[1\rightarrow3]$ Gal [49]. We have had the experience of culturing multiple clones of transfected J558L whose antibody product had an essentially normal glycoform profile over several years; then, for reasons unknown to us, the antibody product included high mannose oligosaccharides and low site occupancy [Lund, Takahashi and Jefferis, unpubl. obs.]. A similar experience has been reported for human IgG1 and IgG2 antibodies produced by heterohybridomas for which variable proportions of high mannose containing antibody was obtained [50]. Glycosylation appeared to be dictated by the mouse plasmacytoma partner since N-glycolylneuraminic acid but no bisecting GlcNAc was added. Similarly, a humanised anti-CD18 antibody produced in NSO cells was shown to contain five oligomannoside-type structures in addition to the usual biantennary-type oligosaccharide moieties, no bisecting GlcNAc and no sialic acid [51]. The glycosylation status of human anti-D antibody produced by EBV-transformed lymphocytes grown at low density in static culture or high density in hollow fibre bioreactors also demonstrated high levels of galactosylation for antibody produced at low density and a relatively natural profile of glycoforms for antibody produced in the bioreactor [23]. Heterohybridomas secreting anti-D antibody have also been established; however, analysis showed that 12/16 such cell lines had incorporated Gal$\alpha[1\rightarrow3]$Gal epitopes into the antibody [52].

A further rodent/human difference is in the form of sialic acid utilised. Polyclonal human IgG has a terminal N-acetyl neuraminic acid sugar on $\sim$25% of oligosaccharides, by contrast the mouse utilises N-glycolyl neuraminic acid. Interestingly, chimeric mouse/human IgG3 produced in J558L cells was shown to be a mixture of molecules having one or the other derivative. This demonstrates that both transferases are available and that their utilisation is affected by subtle structural effects. A bisecting GlcNAc residue is present in $\sim$10–20% human polyclonal IgG but NSO and CHO cells lack the GlcNAc transferase III enzyme required for its addition. The extensive functional

studies reported for recombinant molecules produced in CHO cells suggested that bisecting GlcNAc has little influence on biological activity, however, the presence of glycoforms with bisecting GlcNAc produced by rat YO cells has been held to account for its beneficial biological activity [26]. These findings point to the need for a productive cell line of human origin, however, none is available that has a high endogenous rate of protein synthesis. Many other vehicles for recombinant protein production are being appraised or under development. The early promise of *E. coli* has not been realised for glycoproteins since bacteria do not have a glycosylation machinery [14]. Experiences with insect cells differ. Thus whilst a mixture of high mannose and complex N-linked oligosaccharides was reported for recombinant human plasminogen, including a fully elongated biantennary form (28%) [53] the conclusion drawn from a study of the N-glycosylation of a virion protein was that insect cells were not capable of elongation with the addition of galactose and sialic acid [54]. It is evident that this system is very sensitive to culture conditions and the timing of infection with baculovirus. It is unlikely that other expression systems, such as transfected potatoes, tomatoes, are likely to allow production of glycoproteins that will be acceptable for therapeutic use; it should be remembered that glycosylation is only one of several post-translational events that are essential to the synthesis of a natural form of proteins and glycoproteins.

Whilst one might attempt to develop optimal growth conditions for basic scientific studies, it is likely that they would be too costly to translate into commercial production protocols. Ideally, one would aim for a system that mimics in vivo conditions (homeostasis!) as closely as possible with the maintenance of nutrient concentrations, oxygen tension, removal of metabolites. An unknown factor is the presence of essential growth factors (cytokines) in vivo. A major consideration for biotechnology companies is the overall cost of production and an important element in its determination is downstream processing. Isolation and purification is simplified by the use of defined media and there has been a sustained development of serum-free media with most companies adopting their own undisclosed formulation. Large-scale production facilities have employed air-lift fermenters of 10–12,000 litres capacity. There is a gradual scale-up with the growth of a 'charge' for the next fermenter to allow exponential growth. At the end stage, the cells exhaust the medium, die and protein is released following rupture of the cell wall.

Hollow-fibre bio-reactors have been used for research and intermediate scale production of glycoproteins, including antibodies to be used as in vivo therapeutics. This system does allow continuous exchange between the medium that the cells are suspended in and the 'external' circulating medium. However, the cells are not homogeneously dispersed throughout the cell compartment

but grow in clumps of solid tissue with the result that mass transfer across such a tissue is inefficient and necrosis follows.

We have commented on the heterogeneity of glycosylation of IgG produced in vivo by healthy human adults and instanced altered galactosylation patterns in certain inflammatory diseases. It remains to be determined whether the IgG producing plasma cells are or are not abnormal per se or are developing and producing IgG in an abnormal environment. A fundamental question is whether this is a disease-specific phenomenon that has direct implications for cause and progression or an epiphenomenon that may be used to monitor disease activity and may have value as a prognostic indicator, as for α1-acid glycoprotein [55]. Analysis of mouse and human monoclonal IgGs has demonstrated that each clone exhibits a unique Fc glycosylation profile and, therefore, that the profile for polyclonal IgG is the sum of the many contributing clones. The human monoclonal IgG proteins have been isolated from sera of patients with the disease multiple myeloma. Analysis of a panel of IgG paraproteins with multiple examples of each subclass revealed a subtle template direction effect such that the apparent preference, in polyclonal IgG, for galactosylation of the α[1→6] arm over the α[1→3] arm was reversed for IgG2 proteins and for 2/3 IgG3 proteins [6]. In an extension of these studies, we have observed the α[1→3] preference for a further five IgG3 paraproteins whilst the polyclonal IgG isolated from the same serum sample demonstrated the opposite preference [56]. A further observation is hypogalactosylation of both the polyclonal and the monoclonal IgG, relative to normal polyclonal IgG; however, this did not correlate with IL-6 levels in the same serum sample. It has been shown that IL-6 is a growth factor for plasma cells proliferating in the bone marrow in this disease and that it is reflected in increased IL-6 levels in the serum [57].

For proteins with multiple glycosylation sites, fidelity is observed for the type of oligosaccharide attached at each. Such template direction, excluding outer arm sugars, is exemplified for IgM, IgA, IgE and IgD molecules. Thus, for a mouse IgM secreting plasmacytoma, the oligosaccharide attached at Asn171 is a biantennary complex form, oligosaccharides at Asn332, 364 and 402 are triantennary and at Asn563 predominantly chitobiose ($Man_3GlcNac_2$) [58]. Similarly, in the human IgD molecule an oligomannose form is attached at residue 354 within the C_H2 domain, and complex forms at residues 445 and 496 within the C_H3 domain [59, 60]. While large-scale steric effects have been invoked in influencing accessibility of glycosylation sites to glycosylation enzymes, it is less widely appreciated that smaller-scale changes can also affect glycan synthesis. Repositioning of a carbohydrate attachment site within the Fab region of an antidextran antibody by two residues from Asn58 to Asn60 [9], resulted in the attachment of oligomannose forms in place of a complex form and was accompanied by ≥3-fold reduction in affinity for antigen. This

study employed mouse hybridoma cells for antibody production and noted that the Fab oligosaccharide was more fully processed than the Fc moiety and that Galα[1→3]Gal was added to a significant proportion of Fab oligosaccharide. A more subtle influence of glycosylation status has been demonstrated for a human hybridoma that has a glycosylated λ-chain; variations in glucose availability were shown to determine the size of the oligosaccharide attached and the antigen-binding activity [61]. Alternatively, it has been shown that glycosylation sequons can be introduced into variable regions with consequent glycosylation that does not affect antigen binding and which can be used for conjugation of haptens [62].

The extensive interactions between the oligosaccharide and protein moieties suggest the possibility to modulate them by selected amino acid replacements with a consequent influence on biological activity. Such an effect has been observed on replacement of the core contact residue Asp265 by Ala (DA265), resulting in greatly increased levels of galactosylation but a diminution of FcγRI-mediated function; 50% more oligosaccharide chains with galactose residues on both the α[1→3] and α[1→6] arms for mouse/human chimeric IgG3 produced in CHO cells [63].

In conclusion, it is evident that post-translational glycosylation of proteins can have subtle and more far-reaching structural and functional consequences. These consequences will be particularly manifest for recombinant glycoproteins produced in vitro but intended for in vivo application. A second rapidly developing area of interest results from the observation of altered glycosylation states for specific proteins correlating with disease and/or disease activity. The field is set to expand as sensitive technologies for determining oligosaccharide structures and profiles are now commercially available.

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